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SEED DISINFECTION FOR PURE CULTURE WORK

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In recent years the growing of seed plants in pure cultures for both physiological and pathological purposes has become recognized as an important research method. When parasitic fungi are being studied in relation to their hosts it is necessary to control as many conditions as possible, and especially is it essential to prevent the entrance of other parasites. Moreover, when the relations of higher plants to organic nutrient solutions are being investigated, it is usually necessary to eliminate the possible action of all microörganisms. In order to obtain plants free from bacteria and fungi many and varied methods have been advocated. It is obviously necessary to begin with pure seed or with disinfected seed. This paper deals only with the latter possibility in the establishment of pure cultures.

An examination of the literature indicates that many germicides have been tried in an effort to disinfect seed. In most cases, the investigator has selected some particular germicide, which had proved favorable in one or more cases, and has attempted to make it applicable to all seeds under all conditions. Accordingly, when others attempt to use the method under different conditions poor results are obtained and the method may be considered a failure. There is much dissatisfaction, therefore, with any single germicide as an agent which will disinfect all seed under all conditions.

The chief observation that suggested a further test of disinfectants was the difficulty experienced, at the Missouri Botanical Garden, in obtaining consistent results in regard to seedlings relatively free from contamination for certain "immunity" experiments in which the author was associated with Dr. Duggar. It was found that seeds of the same varieties grown in different parts of the country had to be treated differently and in some cases with a different germicide. This is perhaps only what is to be expected when one realizes that various soil bacteria and fungi show a wide range of tolerance towards the same toxic agent. A good example of this is cited by Makemson ('17) in his work with *Cladosporium fulvum.* He found that the spores of this fungus would germinate in a relatively strong solution of Bordeaux mixture in a hanging-drop culture. He noted further that under the conditions just mentioned the germ tubes grew as rapidly as in the control. On the other hand, the toxicity of Bordeaux mixture for most fungi is well known.

Many species of *Fusarium* are able to grow in a 1 per cent copper sulphate solution. Young and Cooper ('17), in their work on the determination of fungicidal coefficients, found that *Endothia parasitica* was killed at a dilution of 1 to 16,000 commercial lime-sulphur solution, while it required a 2 per cent solution of copper sulphate to kill this organism, both fungicides acting for a period of 15 minutes. On the other hand, when *Glomerella cingulata* was used and the same time interval employed, a dilution of 1 to 9600 commercial lime-sulphur was necessary to kill the spores, while a 1 per cent solution of copper sulphate was lethal. Lime-sulphur, therefore, is almost twice as toxic to the spores of *Endothia parasitica* as to those of *Glomerella cingulata.* The reverse is true in the case of copper sulphate. Horn ('04) found, in his work with some of the *Phycomycetes,* that exceedingly small traces of $CuSO_4$ would prevent growth. The success of the fungicide, then, must depend upon the species of bacteria or fungus present on the seed.

The nature of the seed will affect the selection of a germicide, since the resistance of different seed is as variable as that of spores. With respect to different germicides it is known that seeds may exhibit marked differences in permeability. The moisture content of the seed will also probably modify its resistance to any injurious agent. This fact has been frequently pointed out, and recently clearly demonstrated by Coons and McKinney ('17) in their work on the treatment of grains for smuts, using formaldehyde as the disinfectant. They found that wheat seed high in moisture content absorbed the formaldehyde vapor very rapidly and were materially injured. Many other examples might be cited which, up to the present time, have seemed to indicate that no one germicide can disinfect under all conditions.

In his studies on the comparative viability of seed, fungi, and bacteria, de Zeeuw ('12) was unable by the usual method to free seeds of a certain amount of the disinfecting agent. He believed that the adherence of the agent permitted the continuance of antiseptic action, leading to a false sense of security at the time of making the transfer of the young seedling. He therefore devised a special apparatus by which it was possible to wash the seed thoroughly in order to free it from the germicide, and at the same time it prevented possible contamination from the outside during treatment.

The object of this work is to determine the effectiveness of some of the well-known disinfectants in relation to particular groups of seeds. No new methods have been devised nor new germicides used. In all cases the antiseptic action (that is, antiseptic action due to incomplete washing) of the disinfectant was prevented only when it hindered normal germination and growth of the seed. The percentage of germination and appearance of growth were the indicators in each experiment.

The first germicide employed in this work was commercial chloride of lime (largely calcium hypochlorite), as suggested by Wilson ('15), and his method was followed, namely, "Ten grams of commercial chloride of lime (titrating 28 per cent chlorine) is mixed with 140 cc. of water. The mixture is then allowed to settle for five or ten minutes and the supernatant liquid decanted off or filtered. The solution or filtrate, which contains about 2 per cent chlorine, is used as the disinfectant. The volume of the solution employed should be about five times or more the volume of the seed."

The seeds were placed in glass tumblers, in quantity not to exceed about one-fifth of the final volume. The tumblers were then filled with the above-mentioned filtrate and covered with the top of a sterile Petri dish. The seeds were then removed at intervals of 2, 6, 11, and 20 hours. This was done with sterile forceps in the case of large seed and by means of a platinum spoon with small seed. In this case it was only necessary to drain the seed of the hypochlorite solution, as the antiseptic action did not hinder the germination and development of the seed. The transfer was made to small flasks or Petri dishes (depending on the size of the seed). In these flasks and Petri dishes there had been previously placed a small amount of a synthetic nutrient medium[1] in 1.5 per cent agar. Usually from 10 to 20 seeds were placed in each vessel. The flasks and Petri dishes were then placed in an incubator maintained at 25° C. The varieties of seed used in this experiment are given in table I. Not many kinds could be tried, but it will be noted that those experimented upon represent seeds of widely different families. The seeds were obtained from the Vaughan Seed Company, Chicago, Illinois, in all cases except where note is made to the contrary. More uniformity in results was obtained when seeds were secured from the same company. The results of the disinfectant are shown in table I.

According to the results indicated in the table, the commercial "chloride of lime" was very effective for sugar beet, cucumber, and lettuce, a higher percentage of germination and more vigorous seedlings being obtained by the use of this disinfectant. The most effective time intervals are shown in table IV. However, many seeds of other plants were obtained free from contamination, but the effectiveness of the germicide was not sufficiently high for practical purposes. Certain cucumber seed obtained from other seed companies could not be sterilized even with this disinfectant. Bacteria would continue to grow at the micropylar end of such of these seed as

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[1] The nutrient medium was made up as follows:

Ammonium nitrate	1	gm.
Dihydrogen potassium phosphate	.5	gm.
Magnesium sulphate	.25	gm.
Iron chloride	Trace	
Cane sugar	5	gms.
Distilled water	500	cc.

TABLE I

TREATMENT OF SEEDS WITH CALCIUM HYPOCHLORITE SOLUTION

Seed	Time of treatment (hrs.)	Germination (per cent)	Contamination after 48 hrs. (per cent)	Condition of seedling	Seed	Time of treatment (hrs.)	Germination (per cent)	Contamination after 48 hrs. (per cent)	Condition of seedling
Cucumber (*Cucumis sativus*)	2	95	20	Good	Cotton (*Gossypium*)*	6	85	100	Normal but seed covered with Fusarium
	6	95	15	Good		11	60	100	
	11	95	0	Very good		20	60	100	
	20	95	0	Injured		44	0	100	
Bean (*Phaseolus vulgaris* var. *nanus*)	2	45	20	Fair	Corn (*Zea Mays*) †	2	90	60	Good
	6	16	0	Poor		6	90	20	Good
	11	16	0	Poor		11	80	10	Good
	20	10	0	Poor		20	20	0	Poor
Radish (*Raphanus sativus*)	2	80	50	Growth inhibited	Alfalfa (*Medicago sativa*)	2	80	40	Good
	6	24	40			6	80	20	Good
	11	20	40			11	80	5	Good
	20	20	30			20	40	0	Growth inhibited
Lettuce (*Lactuca sativa*)	2	96	0	Good	Turnip (*Brassica Rapa*)	2	80	40	Good
	6	96	0	Good		6	80	10	Fair
	11	50	0	Poor		11	25	5	Growth inhibited
	20	0	0	Poor		20	0	0	Growth inhibited
Sunflower (*Helianthus annuus*)	2	90	100	Mouldy	Vetch (*Vicia sativa*)	2	90	20	Fair
	6	90	100			6	90	10	Growth inhibited
	11	90	100			11	40	0	
	20	60	100	Poor		20	0	0	
Sugar beet (*Beta vulgaris*)	2	40	10	Normal	Cucumber (*Cucumis sativus*) †	2	60	60	Normal
	6	40	0	Normal		6	60	30	
	11	40	0			11	40	10	
	20	25	0	Injured		20	20	5	

* Seed from Alabama. † Seed from St. Louis Seed Co.

remained viable after 20 hours' treatment with the hypochlorite solution. This, however, either indicates that disinfection did not occur or that the bacteria were shielded by being so far within as to be beyond the range of diffusion of the disinfectant. This disinfectant, then, is only effective with certain seeds, but by extensive tests many more might be added to the list.

Inasmuch as it is not possible to include a test which is in every sense a control, it may be well to state at this point that the germination of all seed was tested out under usual conditions for germination and in every case ranged from 95 to 100 per cent.

The second disinfectant employed was formaldehyde. It is especially toxic to many parasitic fungi inducing plant diseases. In practically all cases where it has been unsuccessfully used as a general germicide, the failure has been due rather to its injurious action upon the seed than to ineffectiveness in controlling fungi and bacteria. Seed injury has been especially noticeable where the gas method has been used. If the seed contains a large amount of water, the gas is absorbed more readily and the seed is promptly injured.

The method employed was the same as the previous one except that the seeds had to be washed free of the disinfectant. It was found after many trials that one thorough rinsing in sterile water was sufficient to remove enough of the formaldehyde to prevent the antiseptic action from affecting the germination and development of the seed. The time intervals for removing seed from the solution were usually 15, 30, and 45 minutes. The effectiveness of the fungicide is shown in table II. Formaldehyde was very effective for a large proportion of the seed used. In most cases, better results were obtained when the seeds were left in a stronger solution a shorter time. Excellent results were obtained when the seeds were first dipped momentarily in 70 per cent alcohol, or for a definite preliminary period in hydrogen peroxide. Where the seeds are fairly resistant this method of treatment is the logical one. Only fair results could be obtained with either cotton or potatoes. In the case of the former, the seed had to be delinted with concentrated H_2SO_4 and then subjected to the formaldehyde treatment. In the case of the potato it was difficult to prevent bacterial growth. Eyes with a very small portion of the potato (1 c. cm.) were washed for twelve hours,

TABLE II
TREATMENT OF SEEDS WITH FORMALDEHYDE

Seed	Time of treatment (minutes)	Germination (per cent)	Contamination (per cent)	Condition of seedling	Seed	Time of treatment (minutes)	Germination (per cent)	Contamination (per cent)	Condition of seedling
2 per cent solution					3 per cent solution				
Dwarf bean	15	96	20	Normal	Dwarf bean	15	96	40	Normal
	30	80	20	Normal		30	98	0	Good
	45	80	10	Normal		45	40	0	Fair
Cucumber	15	92	15	Normal	Cucumber	15	90	10	Normal
	30	80	5	Fair		30	96	0	Normal
	45	60	0	Poor		45	96	0	Poor
Lettuce	15	96	50	Fair	Cotton	15	60	80	Poor
	30	50	0	Poor		30	40	80	Poor
	45	10	0	Very poor		45	40	70	Poor
Radish	15	90	5	Good	Cotton¶	15	60	80	Normal
	30	45	0	Fair		30	60	80	Normal
	45	30	0	Very poor		45	50	5	Normal
	60	4	0	Very poor		60	40	0	Injured
Sugar beet	15	40	5	Very poor	Sunflower	15	90	80	Fair
	30	0	0	Very poor		30	96	70	Fair
	45	0	0	Very poor		45	70	40	Poor
Sunflower	15	85	50	Normal	Sunflower**	15	96	5	Good
	30	66	15	Normal		30	96	0	Good
	45	55	15	Normal		45	70	0	Growth inhibited
Wheat*	15	90	5	Very good	Wheat††	15	96	15	Good
	30	96	0	Very good		30	96	0	Good
	45	92	0	Very good		45	90	0	Fair
Barley	15	80	10	Fair	Alfalfa	15	90	20	Normal
	30	20	5	Poor		30	90	0	Normal
	45	0	0			45	60	0	Injured
Corn	15	90	20	Good	Corn	15	90	0	Normal
	30	90	10	Good		30	90	0	Slightly injured
	45	60	0	Fair		45	40	0	Injured
Garden pea	15	90	20	Normal	Potato	15	100	90	Normal
	30	80	0	Normal		30	100	90	Normal
	45	40	0	Injured		45	90	80	Normal
						60	90	80	Normal

* Preliminary treatment—soaked in water 12 hrs.

** Preliminary treatment—soaked in water 18 hrs.

¶ Preliminary treatment—soaked in water 18 hrs., heated to 68° C. for 15 min.

†† Preliminary treatment—soaked in water 6 hrs.

TABLE II (continued)

Seed	Time of treatment (minutes)	Germination (per cent)	Contamination (per cent)	Condition of seedling	Seed	Time of treatment (minutes)	Germination (per cent)	Contamination (per cent)	Condition of seedling
4 per cent solution									
Cotton	15	60	80	Normal	Potato	15	100	90	Normal
	30	60	60	Normal		30	100	60	Normal
	45	40	20	Slightly injured		45	90	20	Normal
	60	20	20	Injured		60	40	10	Injured
Cotton†	15	60	40	Normal	Potato‡‡	15	100	90	Normal
	30	60	10	Normal		30	100	40	Normal
	45	40	0	Slightly injured		45	80	10	Normal
	60	20	0	Injured		60	40	10	Injured
Cotton‡	15	60	40	Normal	Potato||||	15	100	40	Normal
	30	50	10	Normal		30	100	10	Normal
	45	40	0	Slightly injured		45	60	0	Normal
	60	0	0	Injured		60	20	0	Slightly injured
Cotton||	15	60	10	Normal	Sunflower	15	20	0	Injured
	30	50	0	Normal		30	0	0	....
	45	40	0	Slightly injured		45	0	0	....
	60	0	0	Injured		60	0	0	....
Potato 5% HCHO	15	100	50	Normal	Bean	15	90	0	Normal
	30	80	40	Normal		30	90	0	Injured
	45	60	20	Injured		45	20	0	Badly injured
	60	20	10	Injured		60	0	0	....

† Preliminary treatment—soaked in 1% H_2O_2 for 10 min.

‡ Preliminary treatment—soaked in water 18 hrs., then heated to 65° C. for 15 min.

|| Lint removed with concentrated H_2SO_4.

‡‡ Preliminary treatment—cubes treated with 70% alcohol for 1 min.

|||| Preliminary treatment (see text).

then dipped momentarily in 70 per cent alcohol, then into a 1 per cent H_2O_2 solution for 10 minutes, and finally into the 4 or 5 per cent formaldehyde for 45 minutes. The percentage of germination was low, but after sprouts appeared growth was normal. In a few cases, sterile plants could not be obtained, and this was related to the source and kind of potatoes.

Alcohol was the next disinfectant tried. The same method as the foregoing was employed. The seeds were washed once to prevent further injurious action. No table of results is here included, as I was unable to free any seed from bacteria and still obtain fair germination. The same kinds of seeds as were used in table II were employed in this experiment. Weak alcoholic solutions (10–25 per cent) were used for long periods of time and stronger (25–70 per cent) for short periods as the seed would allow. In only a few cases were sterile seed obtained. Alcohol cannot be entirely discarded as a disinfectant, as it is very effective in a subsidiary way, as when seeds are first immersed in strong alcohol and then followed by another disinfectant. It serves to remove air cavities which would not otherwise allow proper contact for the disinfectant.

The next disinfectant used was mercuric chloride. This has also been used very extensively for seed treatment. It is effective in dilute solutions, and for this reason it is necessary to remove practically all the solution from the seed, as its antiseptic action prevents the germination and growth of the seed. Some seeds are very easily injured by dilutions that will not kill certain fungi or bacteria. This is especially true with barley. It is also effectively used as the chief disinfectant when preceded by hydrogen peroxide or alcohol. The method used was the same as given with the previous disinfectants. The seeds were washed thoroughly of the $HgCl_2$, as its antiseptic action was found to be injurious in most cases. I was able to disinfect many seeds with mercuric chloride that could not be freed from contamination by any other disinfectant. Table III shows the results with this disinfectant.

It might be stated again that the seeds used in this table could not in every case be made free from contamination, but when good viable seed were used, a high percentage of the trials proved successful. Many hundred plantlets were obtained from seed disinfected by the above methods. From the data at hand it appears that a previous treatment with a good bactericide, such as alcohol or hydrogen peroxide, is to be desired. There are many seeds that will withstand a suf-

TABLE III

TREATMENT OF SEEDS WITH MERCURIC CHLORIDE

Seed	Time of exposure (min.)	Germination (per cent)	Contamination (per cent)	Condition of seedling	Seed	Time of exposure (min.)	Germination (per cent)	Contamination (per cent)	Condition of seedling
.1 per cent solution					.5 per cent solution				
Corn	1	90	40	Normal	Garden peas	1	96	40	Normal
	10	90	20	Normal		10	96	20	Normal
	20	80	0	Normal		20	90	0	Normal
	40	40	0	Injured		40	80	0	Slightly injured
Field peas	1	96	30	Normal	Dwarf bean	1	94	40	Normal
	10	96	10	Normal		10	94	10	Normal
	20	96	0	Normal		20	70	0	Normal
	40	60	0	Injured		40	30	0	Injured
Wheat	1	90	35	Normal	Garden cress (*Lepidium*) *sativum*)	1	90	40	Normal
	10	10	10	Normal		10	90	20	Normal
	20	20	5	Injured		20	80	0	Normal
	40	0	0	Injured		40	30	0	Injured
Dwarf bean	1	96	60	Normal	Morning glory (*Ipomoea purpurea*)	1	96	20	Normal
	10	96	40	Normal		10	96	10	Normal
	20	60	10	Injured		20	90	0	Normal
	40	20	5	Injured		40	60	0	Injured
Radish	1	90	60	Normal	Nasturtium (dwarf)	1	90	60	Normal
	10	90	50	Normal		10	90	40	Normal
	20	90	20	Normal		20	90	0	Normal
	40	60	0	Normal		40	90	0	Injured
Alfalfa	1	86	70	Normal	Onion*	1	80	90	Normal
	10	78	60	Normal		10	80	40	Normal
	20	70	10	Normal		20	80	0	Normal
	40	60	0	Normal		40	60	0	Injured
Garden peas	1	96	75	Normal	*Lupinus albus*†	1	90	80	Normal
	10	96	70	Normal		10	90	80	Normal
	20	96	40	Normal		20	90	60	Normal
	40	90	20	Normal		40	80	10	Normal
Barley	1	60	75	Normal	*Lupinus albus*‡	1	90	60	Normal
	10	60	5	Normal		10	90	50	Normal
	20	40	0	Injured		20	90	10	Normal
	40	0	0			40	75	0	Normal

* Preliminary treatment—seed soaked in H_2O 12 hrs., then 1% H_2O_2 for 1 min.

† Preliminary treatment—soaked in 70% alcohol for 1 min.

‡ Preliminary treatment—soaked in H_2O_2 for 10 min.

ficiently long treatment of both agents mentioned, and some, such as barley, that are very sensitive and cannot be so treated. I have not been able to free barley from contamination in more than 75 per cent of the seed used. There are many other seeds that are easily killed by any germicide that will be effective against the adhering microörganisms. In conclusion, therefore, it would seem necessary to continue to treat the various seed differently and with different disinfectants. One of the most important considerations is that there are undoubtedly different contaminating organisms.

TABLE IV

SUMMARY GIVING BEST TREATMENTS FOR VARIOUS KINDS OF SEEDS

(For percentage of germination in each case, see Tables I, II, and III)

Disinfectant	Seed	Strength of disinfectant	Time of exposure	Previous treatment
Calcium hypochlorite ($CaOCl_2$)	Cucumber	Full	11 hrs.	None
	Lettuce	Full	2–6 hrs.	None
	Sugar beet	Full	11 hrs.	70% alcohol, 1 min.
Formaldehyde (HCHO)	Bean	2–3%	30 min.	None
	Cotton	3–4%	45 min.	Soaked in H_2O, 12 hrs., lint removed with H_2SO_4
	Corn	2–3%	15 min.	None
	Cucumber	2–3%	30 min.	None
	Alfalfa	3%	30 min.	None
	Sunflower	2–3%	30 min.	Soaked in H_2O, 18 hrs.
	Wheat	2–3%	30 min.	Soaked in H_2O, 6 hrs., 1% H_2O_2, 10 min.
	Potato	3–4%	45 min.	70% alcohol, 1 min.
Mercuric chloride ($HgCl_2$)	Corn	.1%	20 min.	None
	Field peas	.1%	20 min.	None
	Radish	.1%	40 min.	None
	Alfalfa	.1%	40 min.	None
	Barley	.1%	10 min.	70% alcohol momentarily
	Garden peas	.1%	20 min.	None
	Dwarf bean	.5%	20 min.	None
	Garden cress	.5%	20 min.	None
	Nasturtium	.5%	20 min.	Soaked in H_2O, 12 hrs.
	Onion	.5%	20 min.	Soaked in H_2O, 12 hrs., then in 1% H_2O_2, 10 min.
	Lupines	.5%	40 min.	1% H_2O_2, 10 min.; 70% alcohol, 1 min.

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